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Disruption of mevalonate pathway in zebrafish (*Danio rerio*) after chronic exposure to simvastatin: integration of ecological endpoints with key biochemical and molecular markers

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Disruption of mevalonate pathway in Zebrafish (*Danio rerio*) after chronic exposure to Simvastatin: Integration of ecological end-points with key biochemical and molecular markers

Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Abstract

Simvastatin (SIM), also known as a HMG-CoA reductase inhibitor, is among the most prescribed pharmaceuticals for cardiovascular disease preventions worldwide. It has been continuously discharged to aquatic ecosystems, making SIM a compound of emerging concern. Despite the ubiquitous nature of SIM, its potential effects in aquatic non-target organisms are not yet fully understood. Several studies have shown that exposure to SIM, on μg/L range, is able to produce several adverse effects on aquatic organisms, but there is a lack of chronic studies with SIM environmental significant concentrations where aquatic organisms are continuously exposed through their life-cycle.

The aim of this study was to investigate a multi-level biological response on the freshwater teleost fish *Danio rerio* following a chronic exposure (90 days) to SIM concentrations ranging from 12.8 ng/L to 1600 ng/L. We integrated ecological endpoints, i.e. survival, growth, reproduction, and embryonic development, with biochemical markers of lipid content (cholesterol and triglycerides) as well as molecular analysis of transcript levels of key genes involved in the mevalonate pathway (*hmgcra, cyp51*, *dhcr7*, and *srebp2*).

Most of our results exhibited a non-monotonic dose-response curve with significant decrease in cholesterol and mRNA transcript levels of *hmgcra*, *cyp51* and at some extent *dhcr7*, after zebrafish exposure to 64ng/L and 320 ng/L of SIM, followed by an increase to near control levels for 1600 ng/L. The same type of response was observed for anomalies in the embryonic development. Anomalies increased for concentrations of 12.8 ng/L ad 64 ng/L of SIM parental exposure, with a decrease in the incidence of anomalies in the higher concentrations tested. The highest tested concentration, 1600 ng/L, also induced significant alterations at reproductive level, as well as reduced male weight. In addition, our experiment revealed opposite results from those in the literature regarding mRNA transcript levels of *hmgcra* and *srebp2*, which may be due to a longer time of exposure in comparison to previous studies.

These findings have important implications for environmental risk assessment given that aquatic organisms are chronically exposed to SIM levels in the range of those tested here during several generations.

Resumo

Sinvastatina (SIM), também conhecida como inibidora da HMG-CoA redutase, está entre os produtos farmacêuticos mais prescritos para prevenção de doenças cardiovasculares em todo o mundo. Quantidades crescentes de SIM são detetadas em ecossistemas aquáticos, tornando-se deste modo um composto de preocupação emergente. Apesar de a SIM ter uma natureza ubíqua, os seus potenciais efeitos em organismos não-alvo aquáticos não são ainda totalmente conhecidos. Vários estudos demostraram que a exposição a SIM, a concentrações entre os ng e µg / L, é capaz de produzir vários efeitos adversos em organismos aquáticos, no entanto há falta de estudos de exposição crónica com concentrações ambientalmente relevantes onde os organismos aquáticos são expostos continuamente a SIM ao longo dos seus ciclos de vida**.**

O objetivo deste estudo foi investigar respostas biológicas no peixe teleósteo de água doce, *Danio rerio* após uma exposição crónica de 90 dias a concentrações de SIM que variaram entre 12.8 ng/L e 1600 ng/L. Integramos parâmetros ecológicos tais como sobrevivência, crescimento, reprodução e desenvolvimento embrionário, com marcadores bioquímicos (colesterol e triglicerídeos), bem como análises moleculares da expressão de genes chave envolvidos na via do mevalonato (*hmgcra*, *cyp51, dhcr7* e *srebp2*).

Uma grande parte dos nossos resultados exibiram uma curva de resposta não-monotónica com uma diminuição significativa dos níveis de colesterol, assim como da expressão dos genes *hmgcra* e *cyp51* após exposição a 64 ng/L e 320ng/L de SIM, seguida por um aumento para níveis semelhantes ao controlo na concentração mais alta (1600 ng/L). O mesmo tipo de curva foi observado para as anomalias formadas durante o desenvolvimento embrionário. Observou-se um aumento do número de anomalias para embriões em que a geração parental foi exposta a concentrações de SIM de 12.8 ng/L e 64 ng/L de SIM, seguida de uma diminuição da frequência de anomalias em concentrações superiores. Também foi possível observar alterações a nível reprodutivo e da massa corporal em machos na maior concentração testada, 1600 ng/L. O presente estudo revelou resultados opostos à literatura no que diz respeito à expressão génica de *hmgcra* e *srebp2*, que poderão ser explicados com base numa exposição mais prolongada, em comparação com estudos prévios.

Estes resultados têm importantes implicações para a avaliação do risco ambiental, uma vez que os organismos aquáticos se encontram expostos a SIM na natureza durantes várias gerações.

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List of Abbreviations and acronyms

AACT – Acetoacetyl Coenzyme A thiolase

Acet - Acetone

ATV – Atorvastatin

bp – Base pair

bpm – Beats per minute

cDNA – Complementary DNA

Chol - Cholesterol

CYP – Cytochrome P450

CYP51 – Lanosterol 14α-demethylase

DHCR7 – 7-dehydrocholesterol reductase

EC – Effective Concentration

EDTA - Ethylenediaminetetraacetic acid

EE2 – Ethinylestradiol

ER – Endoplasmic Reticulum

EU – European Union

F - Forward

FET – Fish embryo toxicity test

FPPS – Farnesyl diphosphate synthase

HMG-CoA – 3-hydroxy-3-methylglutaryl Coenzyme A

HMGCR - 3-hydroxy-3-methylglutaryl CoA reductase

HMGS - 3-hydroxy-3-methylglutaryl CoA synthase

hpf – Hours post fertilization

LC – Lethal concentration

LC-MS/MS – Liquid Chromatography – Tandem Mass Spectrometry

LDL – Low density lipoprotein

LOEC – Lowest observed effect concentration

MOA – Mode of action

mRNA – Messenger RNA

MS-222 – Tricaine Methanesulfonate

MVA – Mevalonate

n.d. – Non detected

NHS – National Health System

NMDRC – Non-monotonic dose-response curve

PBS - Phosphate-buffered saline

qRT-PCR – Quantitative real time PCR

R - Reversed

rpl8 – Ribosomal protein L8

USA – United States of America

SIM - Simvastatin

SQS – Squalene synthase

SREBP2 – Sterol regulatory element-binding protein 2

SU –Standard Units

STP – Sewage treatment plant

TGL – Triglycerides

WWTP – Wastewater treatment plant

CHAPTER I.
General Introduction

1.1. Environmental impact of pharmaceuticals

1.1.1. Influence of pharmaceuticals' consumption and market growth in the environmental health.

For the last decades, due to the improvement of medical and pharmaceutical sciences, there has been an increase in the life expectancy. Consequently, the percentage of population requiring health care has raised, mainly related to growth of chronic diseases such as obesity, diabetes, cardio-pulmonary dysfunctions, etc., (Arnold et al., 2014; BIO Intelligence Service, 2013; LaLone et al., 2014). In this context, pharmaceuticals have been used in large quantities for the prevention, diagnosis and treatment of such diseases (Azzouz & Ballesteros, 2012).

Since 1990, the EU pharmaceutical market has grown from 48 billion to approximately 242 billion euros in 2014. EU consumption of pharmaceuticals reached 24% of the world' total, making Europe the second biggest consumer, right after the USA. Several studies, performed between 2006 and 2009, revealed that the anti-hypertensors together with the analgesics were the most consumed human pharmaceuticals in Europe (500 Standard Units/*capita*/year), followed by psychoactives (300 SU/*capita*/year), anti-cholesterol or diabetes drugs (150 SU/*capita*/year), and antibiotics (80 SU/*capita*/year) (BIO Intelligence Service, 2013).

In the past, pharmaceuticals were overlooked as pollutants because exposure levels were assumed to be reduced to produce significant effects in non-target organisms (Arnold et al., 2014; Daughton, 2016; EEA, 1999). However, from mid-1990s, a growing attention has been placed in this class of contaminants. Indeed, the detection of pharmaceuticals in the environment has increased in the last years, not only because of the growth of pharmaceutical industry, but also due to the improvement in the sensibility of the chemical quantification analytical methods (LaLone et al., 2014). Most of the pharmaceuticals are detected in the environment at trace levels, generally, concentrations range between ng/L to low µg/L (Arnold et al., 2014; Azzouz & Ballesteros, 2012; BIO Intelligence Service, 2013; Daughton, 2016; Fent et al., 2006). However, since they are bioactive substances, designed to produce biological effects at rather low concentrations, the scientific community is now in broad agreement that pharmaceuticals may pose a considerable environmental risk (BIO Intelligence Service, 2013). In fact, nowadays, these compounds are considered emergent pollutants of concern capable to produce effects at environmentally relevant concentrations in non-target organisms (Arnold et al., 2014; Fent et al., 2006).

1.1.2. Environmental contamination process

There are several ways by which pharmaceuticals can enter into the environment during their life-cycle (Figure 1). Pharmaceutical manufacturing, consumption and waste management are considered the main routes of environmental contamination and the water bodies are especially vulnerable to emissions of this class of contaminants (Arnold et al., 2014; Fent et al., 2006). Pharmaceuticals manufacturing phase at industrial facilities is very controlled with generally low emissions that are considered negligible. However, developing countries have shown to emit significant amounts of pharmaceuticals and their active substances to the environment during this phase (Arnold et al., 2014; BIO Intelligence Service, 2013; Larsson, 2014; Ortiz de García et al., 2013). The main problem lies on the extent of drugs consumption and waste management. Although most EU member states have provided collecting systems for unused pharmaceuticals, a substantial amount of these products are discharged through sinks and toilets or as domestic waste, due to lack of public awareness about environmental problems around this issue. As a result, an average of 50% of the unused medical products are not collected contributing to the occurrence of pharmaceuticals in sewage systems (Azzouz & Ballesteros, 2012; BIO Intelligence Service, 2013; EEA, 2010; Fent et al., 2006). On the other hand, pharmaceuticals cannot be entirely used by organisms and are released into the sewage systems by excretion as mixtures of both native form and active metabolites resulted of its biotransformation (BIO Intelligence Service, 2013; Ebele et al., 2017; EEA, 2010; Fent et al., 2006).

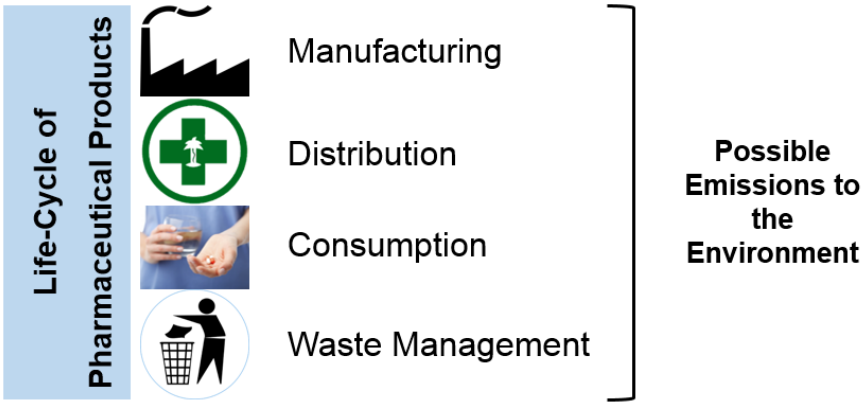

Figure 1. Life-cycle steps of pharmaceutical products

These biologically active compounds reach the sewage systems and are then treated in sewage treatment plants (STP) and/or wastewater treatment plants (WWTP)

(EEA, 2010; Fent et al., 2006). Once in the WWTP, these compounds can be partially removed. However, there is no treatment in these facilities that ensures complete removal of all pharmaceuticals, meaning that low but possibly significant amounts of pharmaceutical active substances are being discharged into surface waters. For this reason effluents from WWTPs are considered one of the most important sources of pharmaceuticals in the aquatic environments (Ebele et al., 2017; Fent et al., 2006; Lapworth et al., 2012). Other pathways for the entrance of pharmaceuticals in the aquatic environment are the sludge spills, the sorption of pharmaceuticals into the activated sludge used as soil fertilizer and veterinary medicines that enter directly or indirectly into the water (Figure 2) (Benotti et al., 2009; BIO Intelligence Service, 2013; Fent et al., 2006; Lapworth et al., 2012).

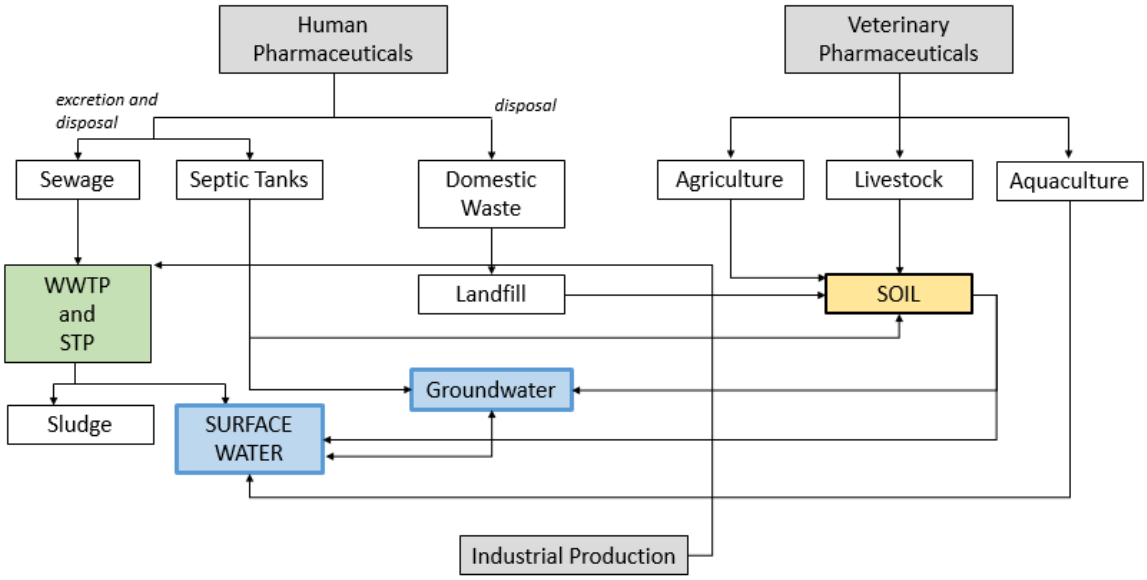

Figure 2. Schematic diagram of sources and pathways of pharmaceuticals in aquatic environment. Adapted from Arnold et al., 2014; BIO Intelligence Service, 2013; Ebele et al., 2017; Lapworth et al., 2012.

1.1.3. Ecotoxicological effects

An inevitable consequence of the worldwide increased consumption of pharmaceuticals over the past decades is the high levels of their discharge into the aquatic environment (Ebele et al., 2017; Lapworth et al., 2012). Although the ecological risk assessment of these chemicals is still on its infancy, a limited number of examples highlight their disruption potential. The best-documented examples of the high impact caused by environmental concentrations of pharmaceutical in ecosystems are the diclofenac and ethinylestradiol (EE2) (EEA, 2010). Diclofenac, an anti-inflammatory drug used in veterinary,

was responsible for 95% decline of the vulture India population due to renal failure (EEA, 2010; Green et all., 2004; Swan et al., 2006). EE2, a synthetic estrogen constitutive of contraceptive pills, was found to be a powerful endocrine disruptor due to its ability to induce feminizing effects in several organisms, particularly male fish, at ng/L concentrations (EEA, 2010; Soares et al., 2009).

Despite these two well documented examples of the ubiquitous nature of pharmaceuticals in the terrestrial/aquatic ecosystems, most of the ecotoxicological studies with pharmaceuticals only report acute toxicity effects. Such data alone are not suitable to address the actual environmental effects of pharmaceuticals, as their concentrations in the wild are too low to pose acute toxicity risk (Dahl et al., 2006, Fent et al., 2006). Furthermore, aquatic organisms are chronically exposed to pharmaceuticals for many generations (Arnold et al., 2014; BIO Intelligence Service, 2013; Fent et al., 2006). Moreover, as previously mentioned, in contrast to other chemicals, pharmaceuticals are designed to be bioactive molecules and hence are expected to target specific signalling pathways (BIO Intelligence Service, 2013; Fent et al., 2006). Taking in consideration that many signalling pathways may be conserved across different phyla, these chemicals may elicit biologically active effects in a broad range of organisms (Fent et al., 2006; Santos et al., 2016). Indeed, one of the current limitations in aquatic toxicity assessment is that the mode of action (MOA) of toxicants is frequently overlooked (Santos et al., 2016). Therefore, there is a need to address the long term-effects of pharmaceuticals as well as identify their MOAs in representative aquatic taxa.

1.1.4. Hypolipidemic pharmaceuticals

Health problems such as overweight and obesity have been increased exponentially over the years, mainly in developed countries (Arnold et al., 2014). These diseases are frequently associated with other health problems, such as elevated plasma cholesterol, coronary heart disease, and consequently the risk of myocardial infarction (Allison & Saunders, 2000; Istvan, 2003; Ribeiro et al., 2015).

Hypolipidemic drugs are divided in two groups, statins and fibrates. Statins are one of the most prescribed pharmaceuticals worldwide for reduction of cholesterol (Al-Habsi et al., 2016; Fent et al., 2006; Santos et al., 2010). These pharmaceuticals are able to decrease the endogenous production of cholesterol by direct competition with the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) (Fent et al., 2006; Key et al., 2008; Santos et al., 2010).

1.1.4.1. Cholesterol, statins and human health

Cholesterol is a 27-carbon, tetracyclic molecule (Burg & Espenshade, 2011) which is synthetized from acetyl – CoA in nearly every cell of the organism. It is essential for many functional processes in living organisms such as structural component of cell membranes, maintaining its stability and fluidity, and also functions as a precursor of all steroid hormones as well as bile acids and vitamin D (van der Wulp et al., 2013). High levels of cholesterol, a medical condition known as hypercholesterolemia, can induce several cardiovascular diseases such as atherosclerosis, stroke and myocardial infarction, as well as type II diabetes (Goedeke & Fernández-Hernando, 2012; van der Wulp et al., 2013). The increased incidence of this type of diseases led to an urgent need of developing pharmaceuticals able to decrease the cholesterol levels. In the 1970s, Akira Endo and his team isolated the first statin, mevastatin, from *Penicillium citrinum*, a potent inhibitor of HMG-CoA reductase (HMGCR), an essential enzyme in the cholesterol biosynthetic pathway, that was able to significantly decrease the cholesterol levels in both experimental animals and patients with hypercholesterolemia (Burg & Espenshade, 2011; Endo, 1992; Endo et al., 1976a; Endo et al., 1976b). This revolutionary discovery allowed treating the high levels of cholesterol in humans. Therefore, several HMGCR inhibitors, i.e. statins, started being developed and by 1990, three new statins, namely lovastatin, simvastatin, and pravastatin, have been approved and marketed in several countries and are still currently used by millions of patients (Endo, 1992). Through the years, statins´ treatment has been shown to decrease and prevent cardiovascular diseases associated with increased lipid content in the bloodstream, demonstrating that the treatment with these pharmaceuticals increase health and quality of life (Grover et al., 2014).

1.1.5. Statins in the mevalonate pathway

Due to its importance for physiological processes, vertebrate cells are able to obtain cholesterol through a combination of dietary source, uptake from the bloodstream, and *de novo* synthesis (Burg & Espenshade, 2011). These cells developed a feedback regulation mechanism in two sequential enzymes, HMG-CoA synthase (HMGS) and HMGCR, in the mevalonate (MVA) pathway in order to achieve a balance between these sources of cholesterol income and avoid overaccumulation (Goldstein & Brown, 1990). Cholesterol is synthesized in the endoplasmic reticulum (ER) and cytoplasm in almost every cells in the organism from acetyl-CoA in the MVA pathway (Goedeke & Fernández-Hernando, 2012). Due to its importance for cholesterol regulation, as well as many other essential molecules resulting from the MVA pathway, it has been exhaustively studied

through the years (Burg & Espenshade, 2011). Cholesterol production from acetyl-CoA requires the action of 20 different enzymes, with HMGCR serving as the primary point of regulation of the entire pathway (Burg & Espenshade, 2011). The upper part of MVA pathway has great importance, since it is the main target of regulation of the whole pathway. The first step involves the synthesis of acetoacetyl-CoA from acetyl-CoA, which then, through the action of the enzyme HMGS, will form HMG-CoA. The binding of HMGCR to HMG-CoA triggers the key reaction of the whole pathway, thus producing MVA (Figure 3). Once MVA is produced, several more reactions occur, giving rise to two branches originated from farnesyl pyrophosphate. Squalene synthesis gives birth to the sterol branch, while the production of geranylgeranyl pyrophosphate lead to the isoprenoid branch (see Figure 3). This second branch produce essential non-sterol molecules, such as heme A, dolichol, ubiquinone and prenylated proteins, which are involved in several biological functions such as growth and metabolism. Endogenous production of cholesterol is also tightly controlled by the sterol regulatory element binding protein 2 (SREBP2), which is involved in the feedback mechanism regulation of this pathway. When cholesterol levels decrease, SREBP2 is activated, increasing the expression of HMGCR as well as low density lipoprotein (LDL) receptors in hepatocyte membranes, thus increasing cholesterol biosynthesis and uptake, respectively (Al-Habsi et al., 2016). The LDL receptors capture LDL cholesterol from the blood, lowering serum LDL cholesterol. This feedback mechanism is also observed after statin treatment, which partly explains how serum LDL cholesterol decrease after statin treatment (Al-Habsi et al., 2016; Altekin et al., 2002; Fent et al., 2006).

For a long time, investigators speculated the precise target of cholesterol biosynthesis regulation. By 1960, Siperstein and Guest (1960) discovered that the conversion of HMG-CoA to mevalonate (MVA), was the responsible reaction for the cholesterol feedback control. It was then discovered that HMGCR was the enzyme responsible for this conversion, and the administration of statins, i.e. HMGCR inhibitors helped clarifying the exact role of this enzyme in the MVA pathway (Eisa-beygi et al., 2013).

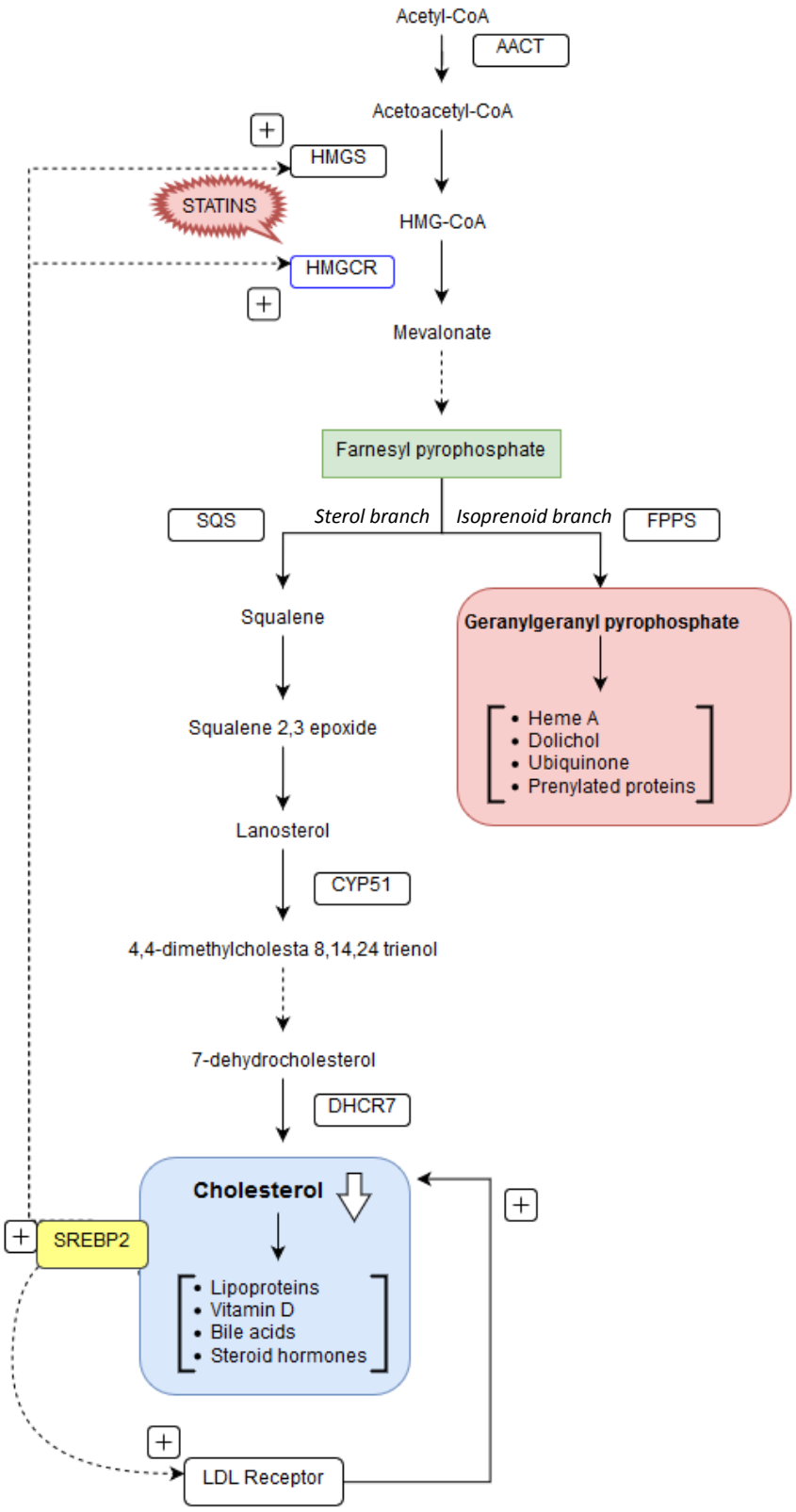

Figure 3. Schematic representation of the interactions of statins in the mevalonate pathway in vertebrates. Statins compete with HMG-CoA for the binding site on HMGCR thereby inhibiting its activity and reducing cholesterol synthesis. SREBP2 is then activated leading to an increase of HMGCR and LDL receptors expression in order to increase cholesterol biosynthesis. Apart from sterol branch (cholesterol synthesis), other isoprenoids (heme A, dolichol, ubiquinone) and prenylated proteins are synthesized via the mevalo-

nate pathway by geranylgeranyl pyrophosphate. Adapted from (Al-Habsi et al., 2016; Goldstein & Brown, 1990; Santos et al., et al., 2016). AACT – acetoacetyl-CoA thiolase; HMGS – 3-hydroxy-3-methylgrutaryl-CoA synthase; HMGCR - 3-hydroxy-3-methylgrutaryl-CoA reductase; SQS – squalene synthase; FPPS – farnesyl diphosphate synthase; CYP51 – lanosterol 14α-demethylase; DHCR7 – 7-dehydrocholesterol reductase; SREBP2 – sterol regulatory element-binding protein 2; LDL – low density lipoprotein.

Statins are hypocholesterolaemic drugs, one of the most prescribed classes of pharmaceuticals in the world (Neuparth et al., 2014; Santos et al., 2016). Statins compete with HMG-CoA for the active binding site in the enzyme HMGCR. Once bound to the enzyme, statins alter its conformation, thereby inhibiting its function (Al-Habsi et al., 2016; Istvan, 2003). Moreover, part of statins structure have a component which resembles HMG-CoA, being yet more hydrophobic and more bulky than the natural substrate (Istvan, 2003). These characteristics make statins affinity to the HMGCR about three orders of magnitude greater than the natural substrate, easily inhibiting the entire pathway by denying the MVA synthesis (Moghadasian, 1999). In addition to the cholesterol lowering action, statin treatment has been shown to have other pleiotropic effects in mammals such as anti-inflammatory, antioxidant, Immunomodulatory, angiogenesis promotion and increase in bone formation (Grover et al., 2014). Evidence of its beneficial effects in the treatment of certain types of cancer and Alzheimer's disease has also been growing (Chen et al., 2016; Schointuch et al., 2014). However, statins have also been shown to produce several undesirable side effects in humans and other mammals. Grover et al (2014) compiled a list of statins adverse effects that included myopathy, hepatotoxicity, diabetes mellitus, erectile dysfunction, prostate cancer and pro-inflammatory, neurologic, pulmonary and ophthalmological manifestations. Due to its involvement on high incidence of rhabdomyolysis, cerivastatin was the first statin removed from European and American pharmaceutical markets in 2001 for safety purposes (Grover et al., 2014). Many of these effects may be a consequence of the inhibition of the upper MVA pathway, which not only block cholesterol production, but also other essential molecules resulting from the isoprenoid branch previously mentioned (Gee et al., 2015; Grover et al., 2014).

Statins are generally rapidly degraded in the liver. This organ is able to metabolize approximately 50 - 95% of the hepatic statins (Evans & Rees, 2002). The main rout of removal involves the activity of Cytochrome P450 (CYP) 3A subfamily. However it has been shown that they can also suffer glucuronidation (Evans & Rees, 2002; Fujino et al., 2004). Although this drug have low systemic availability, due to its rapid metabolization, it has high biological activity since hepatocytes are the main site of action of statins (Evans & Rees, 2002).

1.1.6. Effects of Simvastatin in the aquatic environment

Statins, mainly SIM, are amongst the most prescribed pharmaceuticals in the western countries as primary treatment for hypercholesterolemia by decreasing serum LDL-cholesterol levels (Burg & Espenshade, 2011; Estey, 2007; Neuparth et al., 2014). Annual reports from INFARMED, a Portuguese entity responsible for the supervision of the pharmaceutical sector, indicated that over more than 12 years, SIM has been the most sold statin in the Portuguese national health system (NHS) and its usage has been increasing over the years (Figure 4) (INFARMED, 2017). It is also important to note that SIM introduction in Europe is relatively recent. Taking these facts into account, it is almost certain that SIM increasing sales and continuous emissions will increase its concentrations in aquatic ecosystems (Ribeiro et al., 2015).

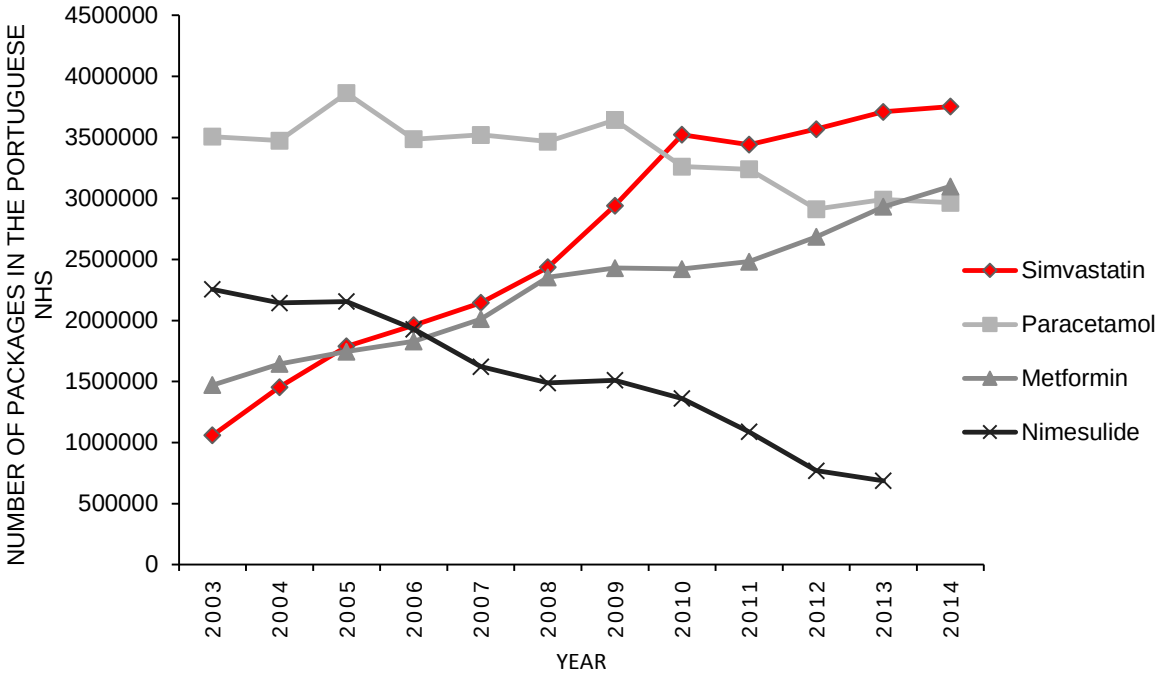

Figure 4. Comparison of the 12 years evolution of number of packages of the main pharmaceutical sold (Paracetamol, Metformin, Nimesulide and Simvastatin) in the Portuguese National Health Service (NHS).

Several authors have reported the presence of SIM in WWTPs worldwide (Table 1). However little information about surface waters concentration is available with a predicted environmental concentration of 369.8 ng/L in Portuguese waters (Pereira et al., 2015). Influent waters of WWTP has been shown to carry high amounts of this pharmaceutical and as a consequence of the lack of efficient water treatments in these facilities,

effluents end up also carrying substantial amounts of this compound. A study performed by Pereira et al. (2016) in several Portuguese WWTPs revealed that in certain cases influents could carry up to 8 900 ng/L of SIM and WWTP effluents ended up discharging 1 500 ng/L of SIM into receiving water bodies (Table 1).

Table 1. Detected SIM concentrations (ng/L) in several WWTP (influents and effluents) across the world. Non detected concentrations are marked with n.d.

Provenience	Detected concentrations (ng/L)	References
WWTP influent	4.0	(Miao & Metcalfe, 2003)
WWTP effluent	1.0	
WWTP influent	1 230.0	(Ottmar et al., 2012)
WWTP effluent	90.0	
WWTP influent	976.0	(Sousa, 2013)
WWTP effluent	197.0	
WWTP influent	8 500.0	(Pereira et al., 2015)
WWTP effluent	369.8	
WWTP influent	40.0	(Verlicchi et al., 2012)
WWTP effluent	n.d.	
WWTP influent	798.0	(Kasprzyk-Hordern et al., 2009)
WWTP effluent	20.0	
WWTP influent	8 900.0	(Pereira et al., 2016)
WWTP effluent	1 500.0	

The main concern of the increase consumption of SIM is its environmental persistence, toxicity, potency, and biological activity in aquatic ecosystems. Furthermore, SIM have a high Log K_{ow} of 4.68, which might indicate that it may bioaccumulate in aquatic organisms (Santos et al., 2016). The fact that SIM is highly lipophilic also makes this statin able to enter tissues of aquatic organisms through direct diffusion, while other statins only enter their target tissues in the liver where receptors are available. Its lipophilic nature is responsible for SIM ability to cross the blood-brain and placenta barriers, which more hydrophobic statins are not able (Moghadasian, 1999).

Santos et al. (2016) demonstrated that the catalytic domains of HMGCR throughout metazoans are highly conserved in respect not only to the interaction with HMG-CoA, but also its interactions with statins. This indicates that statins should be able to inhibit the enzyme HMGCR in several taxa. However we need to be cautious, since several taxa do not show a conservation of its downstream MVA pathways. This means that effects obtained in vertebrates after statin treatment, for example, could not be the same in arthropods, which lack the sterol branch in the MVA pathway (Santos et al., 2016).

Table 2. Simvastatin toxicity data for several groups of organisms. Concentration expressed as µg/L

Organisms	SIM concentration (µg/L)	Duration	Observed effects	Reference
Algae				
Dunaliella tertiolecta	22 800 ([a]EC_{50})	96h	Growth inhibition	1
Arthropods				
Plaemonetes pugio	1 180 ([b]LC_{50}) 1 250 ([c]LOEC)	96h	Decreased larvae survival	2
	10 000(LC_{50})	96h	Decreased adult survival	2
Nitocra spinipes	810 (LC_{50}) 0.16 (LOEC)	Till the 3rd copepodite stage	Decreased growth rate	3
Gammarus locusta	0.32	35 days	Decreased reproductive capability	4
Echinoderms				
Paracentrotus lividus	>5	48h	Decreased larval length	5
	>2	48h	Increase in the percentage of total abnormalities	5
Fish				
Oncorhynchus mykiss	167.4 – 167 429.6	24,48, and 72 h	Cytotoxicity to primary hepatocytes, reduced metabolic	6

			activity and membrane stability	
Danio rerio	837.1	80h	Embryos exhibited developmental arrest, blunted axis elongation, misshapen somites, head and axial necrosis	7
	500	80h	Embryos with increased percentage of total abnormalities.	5

Notes: [a]EC_{50} – median effective concentration; [b]LC_{50} –median lethal concentration; [c]LOEC – lowest observed effect concentration. **References:** (1) - (DeLorenzo & Fleming, 2008); (2) - (Key et al., 2008); (3) - (Dahl et al., 2006); (4) - (Neuparth et al., 2014); (5) - (Ribeiro et al., 2015); (6) - (Ellesat et al., 2010); (7) - (Anderson et al., 2011)

Previous studies have reported detrimental effects of SIM in aquatic organisms at several levels of biologic organization such as impairment of embryo development and disturbances at reproductive level (delay in gonadal maturation, for example), behavioural abnormalities, and even mortality for some vertebrate and invertebrate aquatic organisms (Table 2) (Cunha e al., 2016; Ribeiro et al., 2015; Neuparth et al., 2014; Ellesat et al: 2011; Ortiz de Garcia et al., 2014). Although SIM environmental concentrations generally range from ng/L to μg/L, most of the studies performed regarding SIM toxic potential are based on acute exposures with much higher concentrations than those found in the environment (Table 2).

Studies with other statins have also reported that these drugs were able to decrease cholesterol and triglycerides (TGL) levels in fish species, and modulate expression rates of genes related to cholesterol biosynthesis, as well as genes involved in the biotransformation and efflux of statins (Al-Habsi et al., 2016; Cunha et al., 2016). Larvae developmental arrest and adult sterility in *Caenorhabditis elegans,* were observed after exposure to 558 mg/L of atorvastatin (ATV) (Mörck et al., 2009), the same compound was also able to induce genotoxicity in adult zebrafish (5-14 days; 0.2 – 10 μg/L) (Rocco et al., 2010), and up-regulation of genes involved in oxidative stress response, biotransformation and membrane transport in rainbow trout (Ellesat et al. 2010). The mechanism of action that underlies statins toxicity in non-target organisms is yet not fully understood (Gee et

al., 2015). Chronic toxicity data for SIM is also still very scarce, mainly for aquatic vertebrates. There is an urgent need for the performance of long-term exposure assays integrating several ecological relevant endpoints from individual to sub-individual level which may help clarifying the mechanism(s) underlying statins toxicity to non-target organisms exposed to this compound through their entire life-cycle. In fact, since several studies have demonstrated effects in development and reproduction, these two endpoints should be assessed after a long-term exposure to SIM due to its high importance at population level. Lipids and mRNA contents of genes related to the cholesterol biosynthetic pathway, such as *hmgcr, srebp2* and other ones involved in the sterol branch should also be analysed since almost only acute data is available for these endpoints. A better understanding of the MOA of statins through the assessment of these endpoints after long-term exposure at low concentrations is also needed. The integration of several key endpoints is important to better understand the real implications of chronically exposed aquatic vertebrates to low concentrations in the environment and anticipate effects at population level.

1.2. Model Species: zebrafish (*Danio rerio*)

In order to obtain ecologically relevant data to assess the potential risk of SIM in aquatic ecosystems, one of the key challenges is to choose an appropriate model organism to conduct chronic bioassays. *Danio rerio* is considered a good choice since it is considered a good representative of higher trophic levels in ecotoxicity studies. Over the past thirty years, *D. rerio* has emerged as a vertebrate model for drug development, physiology, genetics (zebrafish genome has been completely sequenced: http://www.ensembl.org/index.html), development, and ecotoxicological studies (Fang & Miller, 2012; Lawrence, 2007). There are several attributes that make this species an ideal model for ecotoxicological studies, offering technical and practical advantages for studying biological processes, effects and mechanisms (Segner, 2009). Fish models are generally considered good organisms to be used in toxicological studies due to their sexual plasticity, easy manipulation and exposure to contaminants in laboratory. The species size, robustness, multiple progeny from a single mating and easy maintenance under laboratory conditions are advantages for its use as bioassay organism (Fang & Miller, 2012; Lawrence, 2007; Soares et al., 2009). In addition, a close phylogenetic relationship between teleost fishes, such as *D. rerio*, and mammals can be observed. Comparison of genome sequences revealed highly conserved genes in several signalling pathways, such as inflammation, oxidation and lipid metabolism (Fang & Miller, 2012). Studies have demonstrated high similarities in the functionality of certain genes and proteins when

compared to its mammalian homologs (Fang & Miller, 2012; Ho et al., 2004). In particular, *D. rerio* have been shown to share a high amount of genetic identity with humans. Almost 70% of human genes have one zebrafish orthologue, facilitating extrapolation of data to other vertebrates (Howe et al., 2013; Santoro, 2014).

Commonly known as zebrafish, *D. rerio* is a small freshwater teleost fish belonging to the Cyprinidae family. This species is native from South Asia, being distributed across parts of India, Bangladesh, Nepal, Myanmar, and Pakistan (Lawrence, 2007). Adult zebrafish can reach up to 30-40 mm long and weight 300-500 mg (Fang & Miller, 2012). When adult, this species present a slight sexual dimorphism upon reaching sexual maturity (Figure 6). Males present a slender figure with a slight yellow coloration on its caudal fin and belly, whereas females have a more silvery aspect and swollen bodies due to their high egg production (Lammer et al., 2009).

Figure 5. Zebrafish (*Danio rerio*) female (A) and male (B).

In laboratory conditions, zebrafish is able to breed through the whole year. Zebrafish spawns at dawn, with a frequency of about three days, and the daily number of eggs laid by a female is quite variable, ranging from 100-500 eggs in each occasion. Spawning occurs in the first 30 to 60 minutes after the beginning of the light period, so large numbers of eggs at similar stages of development are available and easily collected. Since zebrafish embryos develop outside their mothers favours its manipulation and incubation in 24 well plates. In addition, their rapid development, approximately 3 days at 28.5°C, and optimal transparency are ideal for developmental studies and since its stages of development are well known, embryos can be used in toxicity assays (Kimmel et al., 1995; Segner, 2009).

1.3. Objetives

The present work aims at investigating several biological responses in *Danio rerio*, following chronic exposure to environmental relevant concentrations of SIM (ng/L). Here we integrate multiple key endpoints at individual level (survival, growth, reproduction, and embryonic development), which might give insights over possible adverse long term effects at population level, with biochemical markers of lipid homeostasis (cholesterol and triglycerides) and molecular analysis (mRNA levels of *srebp2*, *hmgcra*, *cyp51*, and *dhcr7* genes). These sub-individual endpoints should give more information regarding SIM MOA at relevant environmental concentrations.

The aim is to increase SIM database, in order to better predict potential harmful effects in environmental populations after long term exposure.

CHAPTER II.
Material and Methods

2. Material and Methods

2.1. Chemicals

Simvastatin (SIM, CAS 79902-63-9 - 97.0 % purity) was purchased from Sigma-Aldrich. The SIM stock and working solutions were prepared in acetone (ACET, 99.0 %), purchased from BIOCHEM chemopharma, with a maximum ACET concentration of 0.0002%. The prepared solutions were stored in the dark at -20°C.

2.2. Zebrafish maintenance

Juvenile wild-type zebrafish with 50 days were obtained from Singapore local suppliers. Animals were acclimated to controlled laboratory conditions, in 250 L aquarium with dechlorinated filtered and aerated water. During this period, fish were kept at 28 ± 1°C, photoperiod at 14 h light:10 h dark and fed, *ad libitum,* three times per day with commercial fish diet Tetramin (Tetra, Melle, Germany). These conditions were maintained for 15 days until the beginning of the chronic bioassay.

2.3. Chronic toxicity bioassay

The chronic bioassay were carried out at *Biotério de organismos aquáticos* (BOGA) located at CIIMAR. The experiment was subject to an ethical review process carried out by CIIMAR animal welfare body (ORBEA) prior to the experimental work is started. The bioassay was performed in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes, and the Portuguese 'Decreto Lei' 113/2013.

Juvenile zebrafish were allocated to 30 L aquaria in a flow-through system. The water flow was maintained at 1.08 L per hour using a peristaltic pump (ISM 444, ISMATEC) provided with dechlorinated tap water (carbon activated filtration) and the water level in each aquarium was maintained at 27 L. The bioassay was performed during 90 days. Each aquarium had 25 juvenile zebrafish, with a water temperature of 28 ± 1°C, 14:10 h (light:dark) photoperiod and a mean ammonia concentration of 0.08 ± 0.04 mg/L. Throughout the bioassay, the amount of food distributed was adjusted according to fish development and size. The food consisted in commercial fish diet Tetramin (Tetra, Melle, Germany) twice a day, supplemented with 48 hours live brine shrimp (*Artemia* spp) since one week before the start of reproduction. The experiment consisted of six treatments in duplicate: a control (dechlorinated water); a solvent control (0.0002% ACET), and four SIM treatments: 12.8 ng/L, 64 ng/L, 320 ng/L, and 1600 ng/L concentrations. The selection of these SIM concentrations was based in previous studies (Neuparth el al., 2014),

that are environmentally relevant. A SIM stock solution was prepared in ACET with a concentration of 4 mg/mL and an aliquot of this stock was serially diluted in order to prepare the SIM working solutions. These solutions were stored in obscurity at -20°C. Based on preliminary tests, in order to maintain exposure concentrations, the working solutions were dosed directly into the water twice a day (in the morning - T 0 h - and in the afternoon - T 8 h), in a volume that was equivalent to the water renewal during that period.

2.4. Reproductive capability

Reproductive capability studies were performed 70 days after the beginning of the chronic exposure for all the tested groups. Reproductive success was assessed through the evaluation of two factors: fecundity, i.e. number of embryos per female per day, and percentage of viable/fertilized eggs per female per day. In the afternoon, before the beginning of the trials, each aquarium was divided in two and in each division a suspended cage, with a bottom net covered with marbles, was fixed in order to mimic the natural substrate. Males and females were equally distributed through the cages in order to have the same sex ratio in each treatment (four sub-replicates per treatment) (Figure 6). The Sex ratio was determined by visual observation of each animal which was confirmed at the end of the bioassay, by stereomicroscope gonads observation.

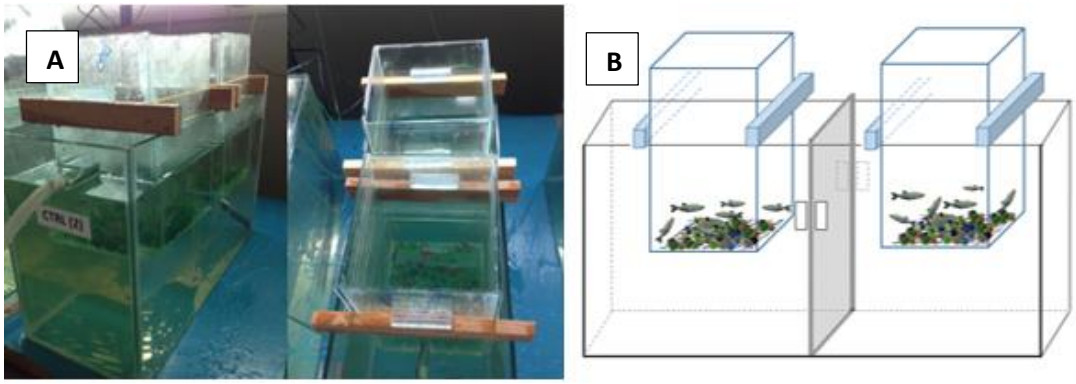

Figure 6. Representation of the breeding setup for reproductive capability assay. A – images of the actual setup and B – schematic representation of the setup.

During five consecutive days, (1-1.5 hour after sunlight) breeding fish were removed, the eggs were collected from the bottom of the tank by siphoning, cleaned and conserved in 70% ethanol for further counting and determination of the percentage of viable eggs (Figure 7).

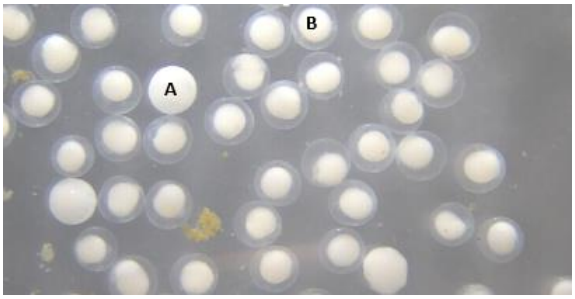

Figure 7. Stereomicroscope view of embryos stored in ethanol 70%. It is possible to distinguish viable embryos (B) from non-viable (A) through its transparency.

2.5. Embryogenesis studies

Embryogenesis studies were carried out based on the most recent OECD guideline for fish embryo acute toxicity (FET) test (OECD test 236, 2013), with slight modifications. During the reproductive trials, 15 embryos with 1-1.5 hpf (hours post fertilization) from each treatment sub-replicate were randomly separated, cleaned and placed in 24 well plates, one per well with 2ml of clean dechlorinated water (Figure 8). The 24 well plates were randomly maintained on a water bath at 26.5 ± 0.5 °C during 80 h. Embryos were checked every day for mortality and water was not changed since it had no contaminants. At the end of the 80h embryos were observed under a stereomicroscope for anomalies detection and abnormal embryos were photographed. Morphological abnormalities on head, tail, eyes or yolk-sac, pericardial edema, abnormal cell growth and developmental arrest were recorded as present or absent. Heart rate was evaluated in four embryos per plate using a stop-watch during 15s, restarting the counting if the embryo moved.

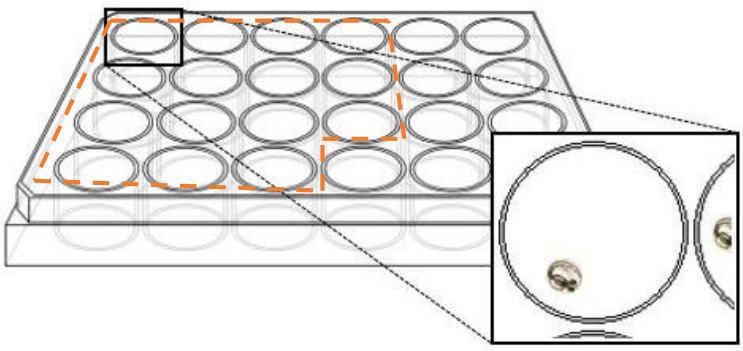

Figure 8. Schematic representation of the disposition of embryos in the 24 well plate.

2.6. Sampling

At the end of the chronic bioassay, animals were sacrificed with an anesthetizec overdose of 300 mg/L tricaine methanesulfonate (MS-222) with the addition of the same amount of sodium bicarbonate, in order to prevent the acidification of the solution and to guarantee minimum pain for the animals. All animals were measured and weighted (Figure 9) for Fulton's condition factor determination, which is calculated from the relation between length and weight according the equation: $K = (weight / lenght^3) \times 100$ (Nash et al., 2006).

Livers, gonads and cerebrum of randomly 15 males and 15 females of each treatment were collected and preserved in RNALater for gene expression analysis. Livers and muscle from the remaining animals were frozen in liquid nitrogen and stored at - 80°C for further cholesterol and TGL quantification.

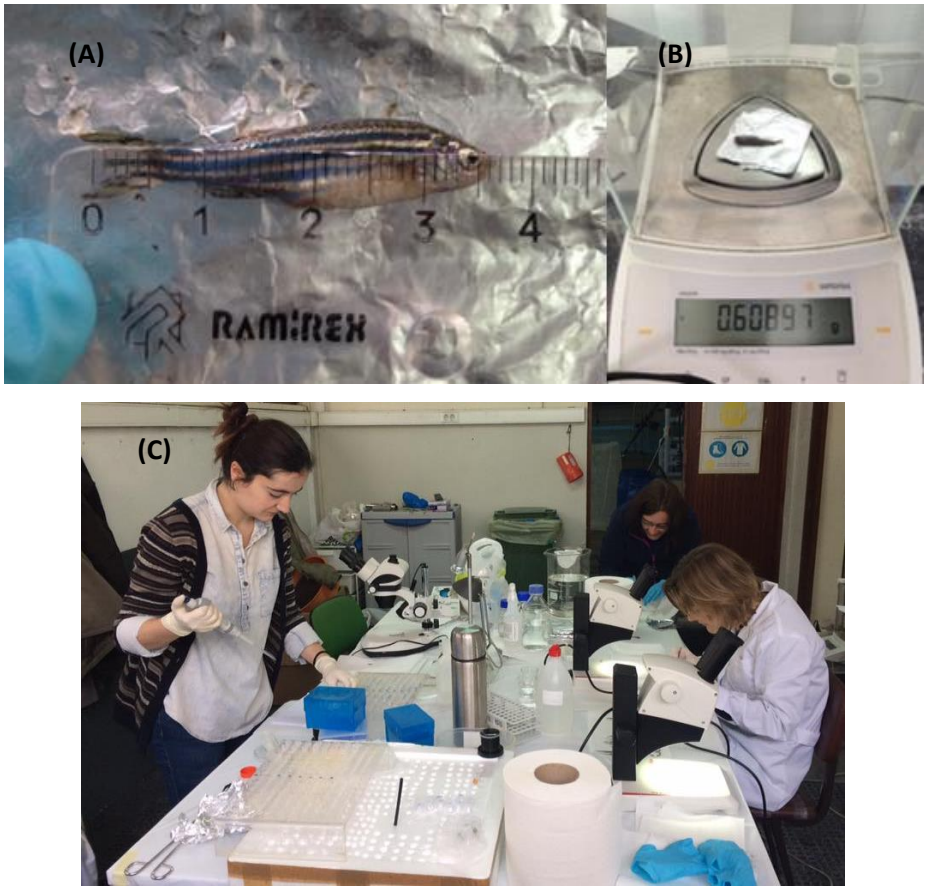

Figure 9. Measurements of zebrafish length (A) and weight (B). Collecting tissues for gene expression and cholesterol/TGL quantification (C)

2.7. Gene expression

2.7.1. RNA isolation and cDNA synthesis

Liver samples of the different treatments, stored in RNALater, were used to isolate total RNA via the Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer's protocol. Approximately 3 mg of tissue was homogenized, with the help of ceramic beads on a Precellys 24 homogenizer to lyse the cells. The homogenate was then filtered to reduce the viscosity and clean the lysate. The RNA was then bonded to the silica membrane with the addition of ethanol (70%), and the addition of DNaseI digested the DNA present in the silica membrane. The silica membrane was then washed with two different buffers in order to remove salts, metabolites and macromolecular cell components. Finally, the membrane contained pure RNA was eluted with RNase-free water.

RNA quantification was performed by the measurement of optical density with a Take3^{TM} on a microplate reader (Biotech Synergy HT) coupled with the software Gen5 (version 2.0). RNA quality was verified by electrophoresis in 1.5 % agarose gel and through the measurement of the ratio of absorbance at λ260/ λ280 nm. All isolated RNA samples were stored at - 80°C until further use. Total cDNA was generated from 1µg of total RNA extracted from the liver using the iScriptTM cDNA Synthesis Kit (Bio-Rad). Due to time constrains, brain and gonads were not processed for genetic analysis.

2.7.2. qRT-PCR

Several enzymes involved in the mevalonate pathway, were selected accordingly to their roles in the cholesterol biosynthesis to evaluate their gene expression in each of the 6 treatments used (control, solvent control, 12.8, 64, 320, and 1600 ng/L SIM concentrations). The 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) was the first gene selected, because it is the responsible for the limiting step in the mevalonate pathway, and statins act as competitive inhibitors of HMGCR. According to the previous studies on the gene tissue distribution, we chosen to assess 3-hydroxy-3-methylglutaryl-CoA reductase a (*hmgcra*) instead of *hmgcrb*, since the first one is predominant in the liver and the second in the brain (Al-Habsi et al., 2016). Other two genes, lanosterol 14α-demethylase (*cyp51*) and 7-dehydrocholesterol reductase (*dhcr7*) were chosen as intermediate genes in the MVA pathway in order to evaluate changes on downstream of HMGCR after its inhibition (Figures 3 and 10). Moreover, mRNA levels of the sterol regulating element-binding protein 2 (*srebp2*) were also assessed to verify if a feedback mechanism carried out by its enzyme would occur.

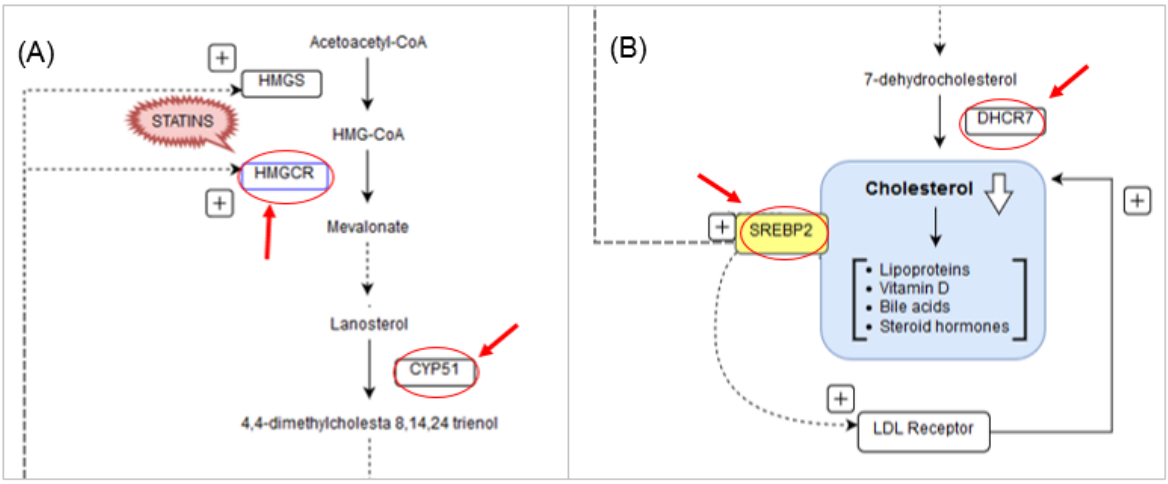

Figure 10. Schematic representation of the sites of action of the selected genes on the MVA pathway. Circles and arrows indicate the target genes. A – upper and middle section of the pathway; B – lower section of the MVA pathway.

Gene expression profiles of *srebp2*, *hmgcra*, *cyp51*, and *dhcr7* were assessed by means of quantitative real time PCR (qRT-PCR). Ribosomal protein L8 (*rpl8*) was used as reference gene, since it expression levels did not change significantly across treatments. All the primers were already described by other authors (Table 3). The cDNA of liver samples of each treatment (n=8) were amplified in duplicated using the Mastercycle ep *realplex* system (Epperndorf) in 96-well optical plates, containing 10 µL of NZY qPCR Green Master Mix (2x) (nzytech), 0.8 µL of each primer (forward and reversed), 2 µL of cDNA at 100 nmol and 6.4 µL of water in order to reach a final reaction volume of 20 µL. On each plate, a nontemplate control was included. In order to determine the efficiency of the reaction, a two-step qRT-PCR was performed: 95°C of initial denaturation for 3 min, followed by 40 cycles of amplification with a denaturation at 95°C for 15 seconds and combined annealing and extension at 58 - 62°C, depending on the pair of primers, for 25 seconds (Table 3). A melting curve (from 55°C to 95°C) was generated in each run to confirm the specificity of the reactions. In order to confirm the product, the PCR products were analysed by electrophoresis in 2% agarose gel to check the presence of single bands with expected size between 134 and 199 bp, depending on the pair of primers (Table 3). The PCR efficiency for the genes of interest and the reference gene was determined by a standard curve, using six serial dilutions of cDNA pools of all samples (from 0.064 to 200 ng of cDNA). The minimum efficiency obtained was 94% (Table 3).

Relative change in transcription abundance of target genes was normalized to rp18 and calculated using the $2^{-\Delta\Delta Ct}$ analysis method. Control expression levels (acetone treatment) were normalized to 1 and data were then expressed as fold changes of the solvent control group.

Table 3. Primers, forward (F) and reversed (R), and parameters used in the qRT-PCR for gene expression quantification in the liver of *D. rerio.*

Gene	Sequence (5' – 3')	Expected band size (bp)	Combined annealing and extension temperature (°C)	Average efficiency (%)	Reference
srebp2	F: GAGATAAAGTGGACCCCATCG R: CAGAAACTCCAGAACCCCAG	134	60	99	(Al-Habsi et al., 2016; Craig & Moon, 2011)
hmgcra	F: TCGTGGAGTGCCTGGTGATTGGT R: TGGGTCTGCCTTCTCTGCTCTCTC	177	62	98	(Mu et al., 2015b)
cyp51	F: GCTCGGAGACACTCAGACACATCTT R: AGCAGAACTGAAGTCAGGCTCATCT	138	60	96	(Mu et al., 2015b)
dhcr7	F: GAGGAGTTCAGGATGGTGCCCGTA R: GTGGACACAGCATAGCCGAGGATG	199	60	94	(Mu et al., 2015b)
rpl8	F: TTGTTGGTGTTGTTGCTGGT R: GGATGCTCAACAGGGTTCAT	136	58	94	(Lyssimachou et al., 2015)

2.8. Lipid extraction and Cholesterol and Triglycerides quantification

Lipids were extracted from the liver samples stored at -80°C using a low toxicity solvent extraction protocol, adapted from Schwartz and Wolins (2007). The tissues were homogenized in 10 mM PBS buffer pH 7.4, containing 10mM EDTA (10 mg of tissue per 1mL buffer with two ceramic beads) on a Precellys 24 homogenizer. 500 µL of homogenate were then transferred, in duplicates, to test glass vials containing 5 mL of isopropanol/hexane solution (4:1 proportion). The samples were vortexed for 1 minute and incubated at room temperature in the dark, with constant shaking for 2 hours. In order to avoid lipid peroxidation, vials were passed always through nitrogen current before closing. After the incubation period, samples were washed with 2 mL of petroleum ether/hexane (1:1) solution. Vials were again vortexed for 1 min and left in the dark, at room temperature, for 10 minutes. The phases were then separated by adding 1 mL of Milli-Q H_2O, vortexed for 1 min, incubated at room temperature in the dark, , with constant shaking for 20 min and centrifuged at 1000 × G, for 10 min. The upper phase containing the lipids was collected into new vials and evaporated to dryness under nitrogen current. Dried extracts were then stored at - 20°C until cholesterol and triglycerides quantification.

Dry extracts were re-suspended in 100 µL of isopropanol and sonicated for 15 minutes at room temperature in ultra-sound bath Bandelin Sonorex RK100H. Quantification of cholesterol and triglycerides was performed through enzymatically colorimetric assays using Infinity Cholesterol Liquid Stable reagent and Infinity Triglycerides Liquid Stable Reagent, respectively, both purchased from Thermo scientiphic, Biognóstica, Portugal and following the manufacturer's protocol. Samples were measured in duplicates and absorbance determined at 490nm using a microplate reader (Biotech Synergy HT) coupled with software Gen5 (version 2.0). A standard curve was performed, in every run, for optimal quantification. Cholesterol and Triolin standards, were prepared and subjected to 6 serial dilutions (from 0.156251 to 5 mg/mL for cholesterol and 0.0625 to 2 mg/mL for triolin).

2.9. Simvastatin analytic quantification

The actual concentrations of SIM was determined in each treatment three times during the bioassay: 30 minutes after the first contamination of the day with SIM (T_0), 8 hours after the first contamination (T_1), and the final one 30 minutes after the second contamination (T_2). Two samples from each treatment (bulk samples from each replicate) were collected and stored at -20°C. The samples were then send to an external laboratory at the University of Santiago de Compostela, Spain to be analysed. These samples are

currently being processed and quantified by Liquid Chromatography - Tandem Mass Spectrometry (LC - MS/MS).

2.10. Statistical analysis

Data obtained from our study were checked for homogeneity of variances (Levene's test) and normality (Kolmogorov-Smirnov test) and subsequently analysed by one-way ANOVA. Post-hoc comparisons were carried out using Fisher's least significant difference (LSD) test. When no differences were found between control and solvent control groups, these were pooled and referred to as control. For the gene expression, the control and solvent control groups were not pooled and all treatments were compared with solvent control group. Significant differences were set as $p<0.05$. All the statistics were computed with Statistica 13 (Statsoft, USA).

CHAPTER III.
Results

3. Results

3.1. Ecological endpoints

3.1.1. Survival, growth and body weight

Figure 11 shows the survival rates, and mean weight and length of zebrafish (Figure 12), males and females following 90-days exposure to 12.8 – 1600ng/L SIM. No significant differences among treatments were observed in the mortality rate, almost 100% of the fish survived at the end of the bioassay (Figure 11). The males average weight was significantly lower in the highest SIM concentration tested (1600ng/L) in comparison with control groups. Males from 1600 ng/L SIM were 6.14% less heavy than males from control groups. In contrast, the weight of females, the length of males and females and the condition factor were not affected by SIM exposure. (Figure 12).

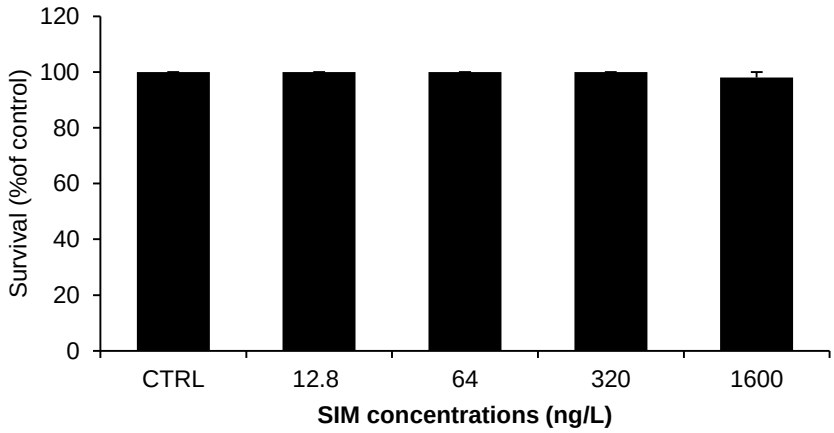

Figure 11. Chronic effects on *D. rerio* survival after 90 days exposure to SIM. Error bars indicate standard errors. As no significant differences were found between control and solvent control groups, the data from these two treatments were pooled and referred to as control.

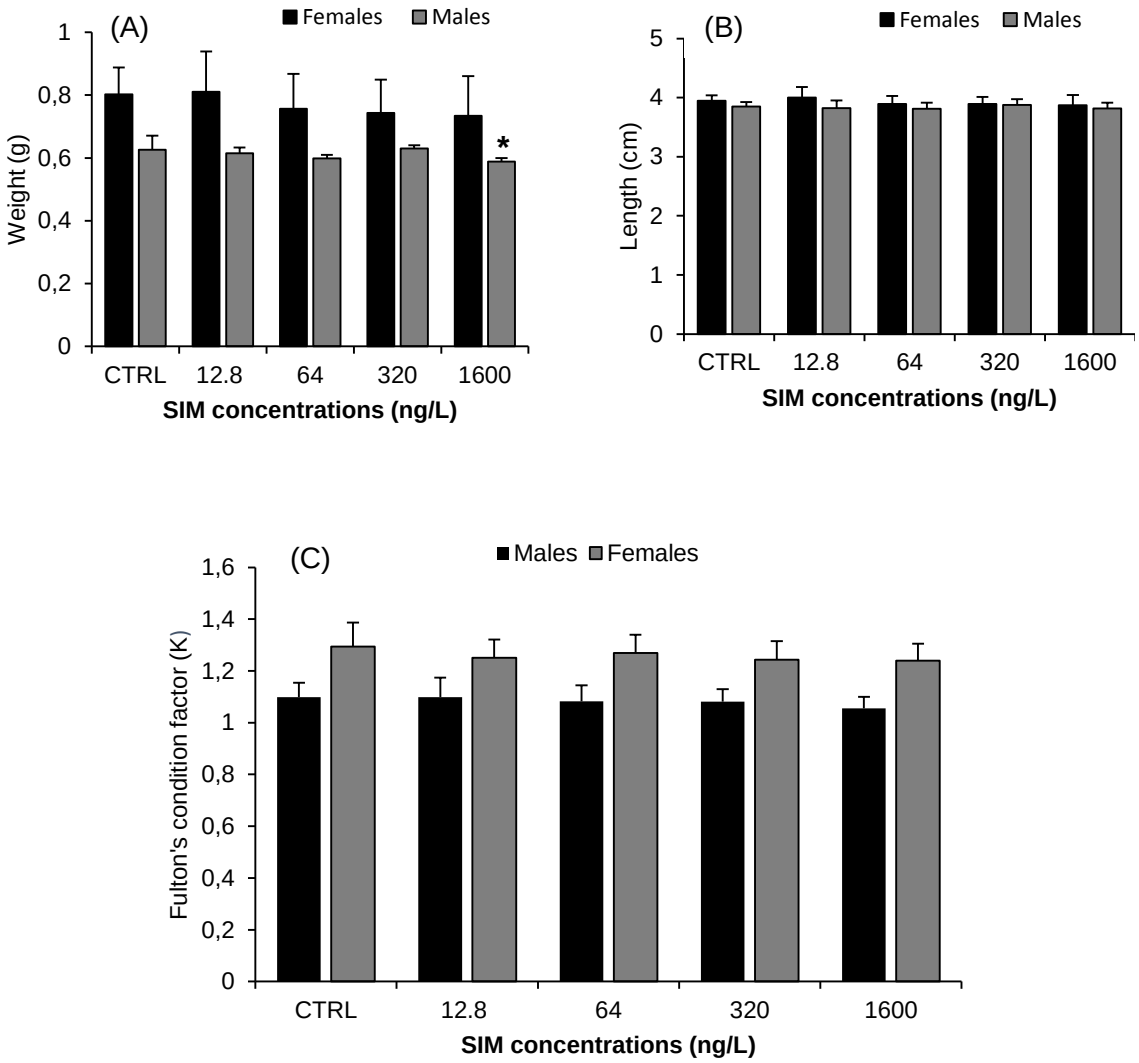

Figure 12. Chronic effects of SIM on weight (A), length (B) and Fulton's condition factor (C) of *D. rerio* after an exposure of 90 days to Simvastatin. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$). As no significant differences were found between control and solvent control groups, the data from these two treatments were pooled and referred to as control.

3.1.2. Reproductive capability

Significant effects on the reproductive capability were observed for fecundity at the highest SIM concentration tested (Figure 13. A). All tested SIM concentrations exhibited an increase in the number of embryos per female per day, however only the 1600 ng/L SIM concentration differed significantly from the control groups. The percentage of fertilized eggs per day (from 92% in control groups to 98% in SIM 320ng/L concentration) did not exhibited significant alterations in any SIM concentrations (Figure 13 .B)

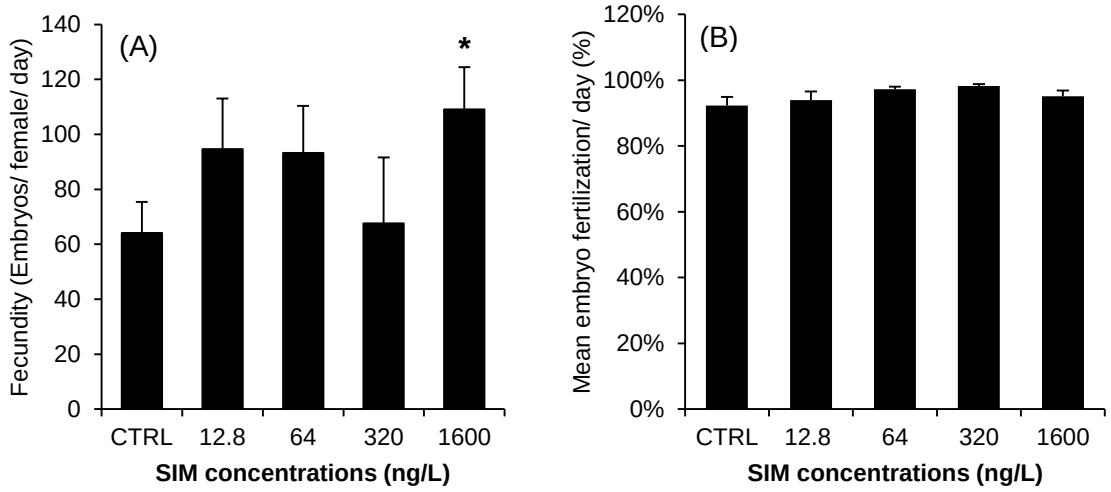

Figure 13. Chronic toxicity effects of SIM on *D. rerio* fecundity (A) number of embryos per female per day, and percentage of fertilized embryos (B) after 90 days of exposure. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$). As no significant differences were found between control and solvent control groups, the data from these two treatments were pooled and referred to as control.

3.1.3. Embryogenesis

After 70 days of SIM parental exposure, the embryos mortality rate at 80hpf ranged from 5% (320 ng/L SIM parental exposure) to 11.7% (1600ng/L SIM parental exposure) with no significant differences among treatments (figure 14. A). From all the abnormalities analysed, arrest in the development and the tail abnormalities occurred in the higher number, therefore we show these abnormalities in individual graphics while the remaining ones accounted for the total abnormalities. Several SIM treatments exhibited developmental arrest (Figure 14. C), however the differences from control groups were not significant. There was a significant increase in the percentage of tail abnormalities in lowest concentrations, i.e. 12.8 and 64 ng/L, with 7.5% increase in both treatments, these abnormalities consisted in shortened and/or curled tails (Figure 15 and Figure 14. B). For total abnormalities (Figure 14. D), the lowest concentration (12.8 ng/L) also exhibited significant differences when compared with control groups with 17.47% increase from control groups. SIM parental exposure was also able to significantly increase heart beat by 8.37 bpm for 64 ng/L of SIM, relatively to control ones (Figure 14. E).

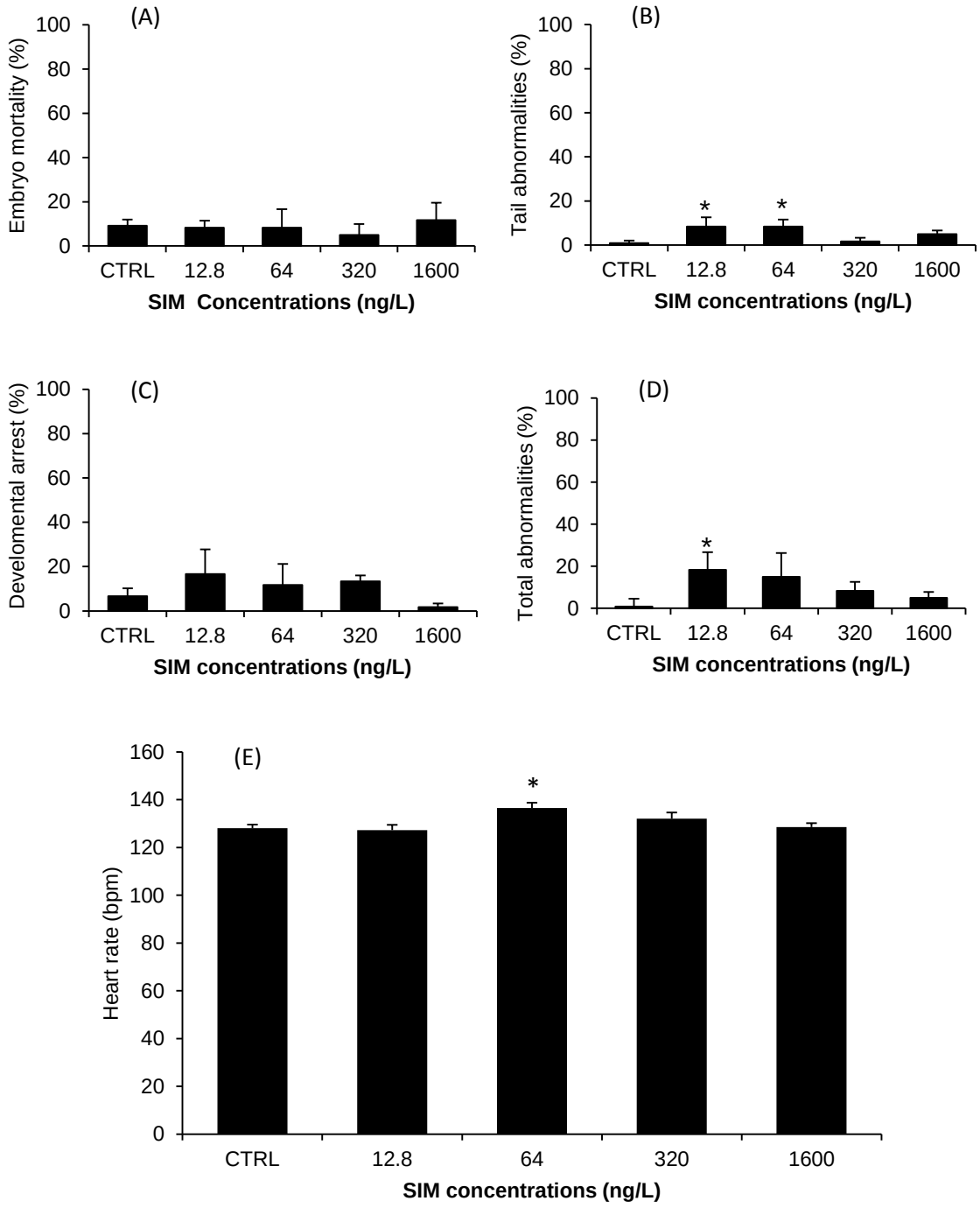

Figure 14. *D. rerio* mortality (A), embryonic abnormalities (B, C and D) and heart rate (E) at 80 hpf, after parental chronic exposure of SIM for 70 days. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$). As no significant differences were found between control and solvent control groups, the data from these two treatments were pooled and referred to as control.

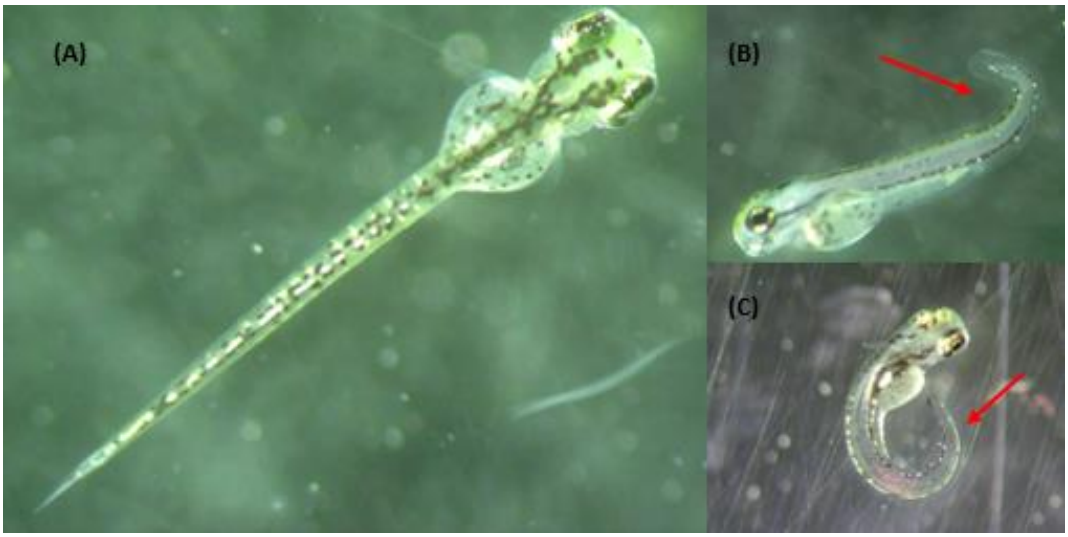

Figure 15. Tail abnormalities, at 80 hpf, after *D. rerio* parental exposure to SIM during 70 days. Comparison between control (A), 12.8 ng/L (B) and 64 ng/L (C).

3.2. Gene expression

Both female (Figure 16) and male (Figure 17) *D. rerio* showed significant differences from the solvent control on their genes expression levels after 90 days of exposure to SIM. However males exhibited more changes than females. *hmgcra* and *cyp51* mRNA expression levels produced non-monotonic dose-response curves (NMDRCs) for both sexes. In females, *hmgcra mRNA* was down regulated by 1.24 and 1.3 fold from the solvent control treatment after exposure to 64 and 320 ng/L of SIM, respectively. Similarly, males also down regulated this gene expression by 1.58 and 1.32 fold in the same treatments, respectively. None of the remaining genes in female zebrafish had their mRNA expression levels decreased, however *cyp51* expression was notably down regulated by 1.2 fold for 64 ng/L, even though it was not significant. Males, on the other hand, exhibited a 1.7 fold significant down regulation when exposed to the same concentration. Male zebrafish also showed significant differences for *srebp2* mRNA expression levels, with a down regulation of 1.48 fold in the higher concentration, 1600 ng/L. Neither males nor females presented significant alterations in *dhcr7* mRNA expression.

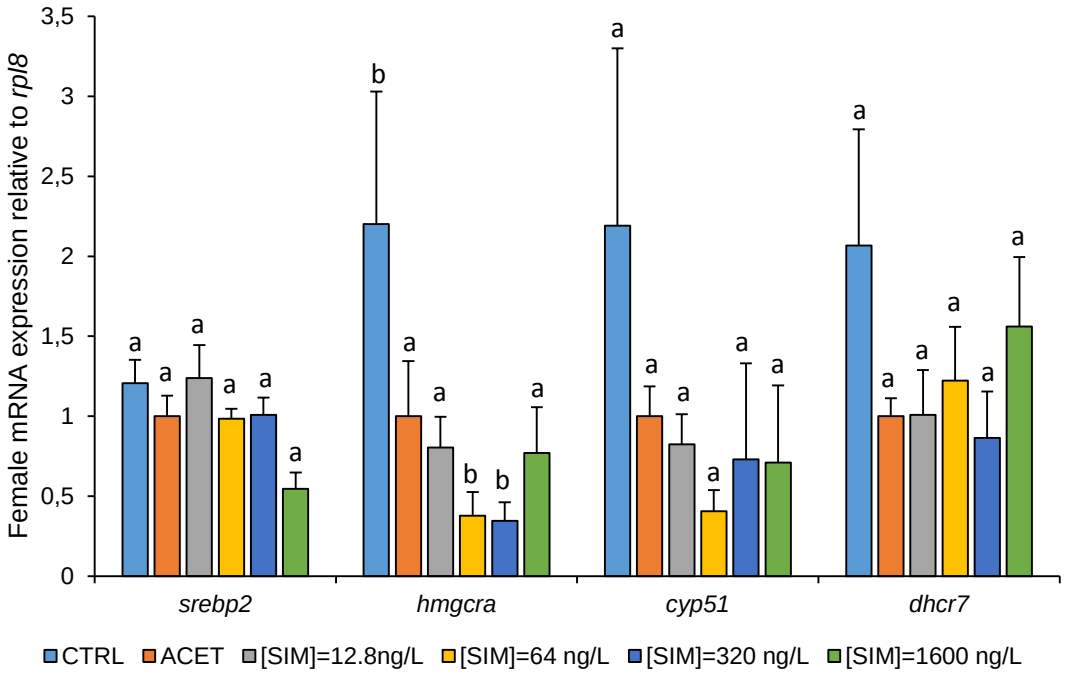

Figure 16. Females' relative mRNA expression of *srebp2, hmgcra, cyp51,* and *dhcr7* in adult *D. rerio* livers after 90 days SIM exposure. Error bars indicate standard errors. Bars with different letters are significantly different from the solvent control treatment (ACET) ($p<0.05$).

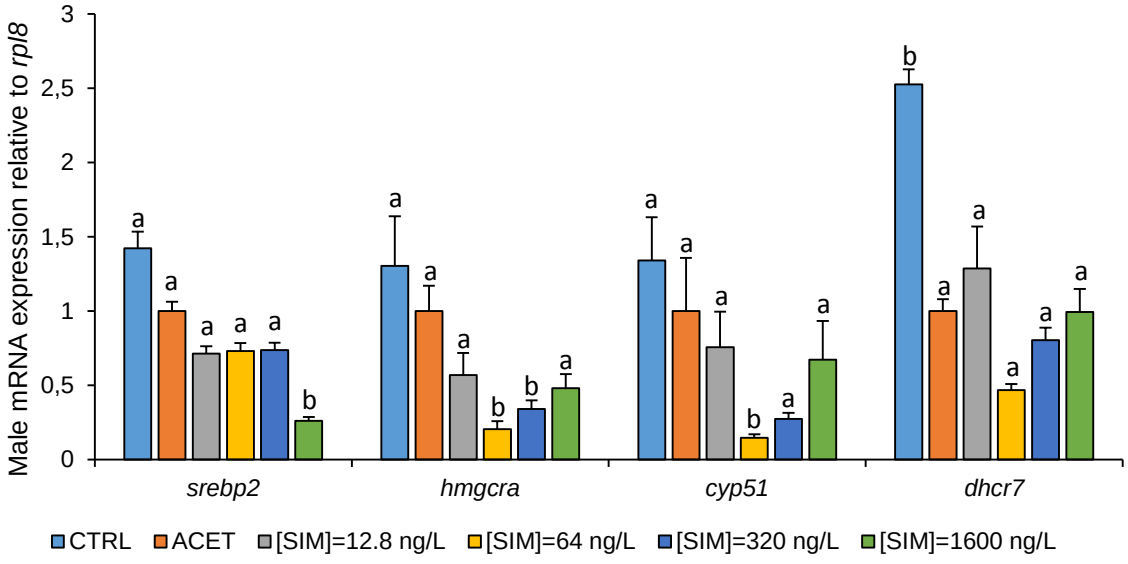

Figure 17. Males' relative mRNA expression of *srebp2, hmgcra, cyp51,* and *dhcr7* in adult *D. rerio* livers after 90 days SIM exposure. Error bars indicate standard errors. Bars with different letters are significantly different from the solvent control treatment (ACET) ($p<0.05$).

3.3. Cholesterol and Triglycerides quantification

Cholesterol (Chol) quantification revealed that, after 90 days of exposure, SIM significantly decreased its liver concentrations in female zebrafish at intermediate concentrations, i.e. 64 ng/L and 320 ng/L with 53.56 and 64.59% decrease from control, respectively (Figure 18). Males also exhibited the same pattern, however it did not shown significant differences from control treatments. Both males and females exhibited a slight increase in the highest concentration when compared with intermediate SIM exposed treatments, yet lower than controls. This biochemical marker developed a NMDRC in a similar way as mRNA transcript levels of *hmgcra* and *cyp51* genes.

Triglycerides (TGL) levels in female liver were significantly lower in all SIM concentrations, with the exception of 320 ng/L (Figure 19). Exposure to 12.8, 64, and 1600 ng/L of SIM was able to decrease TGL levels by 32.59, 26.57 and 45.89 %, respectively. No significant differences were observed in males.

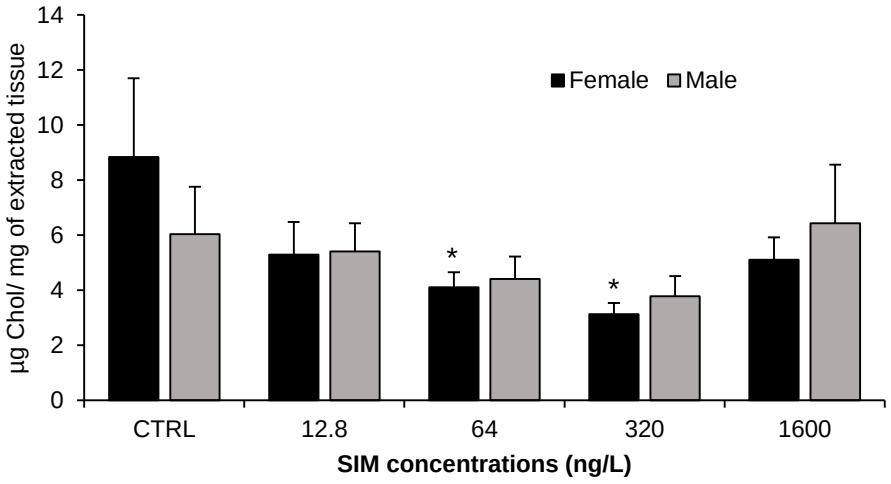

Figure 18. Chronic toxicity effects of SIM on *D. rerio* liver cholesterol content, expressed as µg per mg of extracted tissue, after 90 days of exposure. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$). As no significant differences were found between control and solvent control groups, the data from these two treatments were pooled and referred to as control.

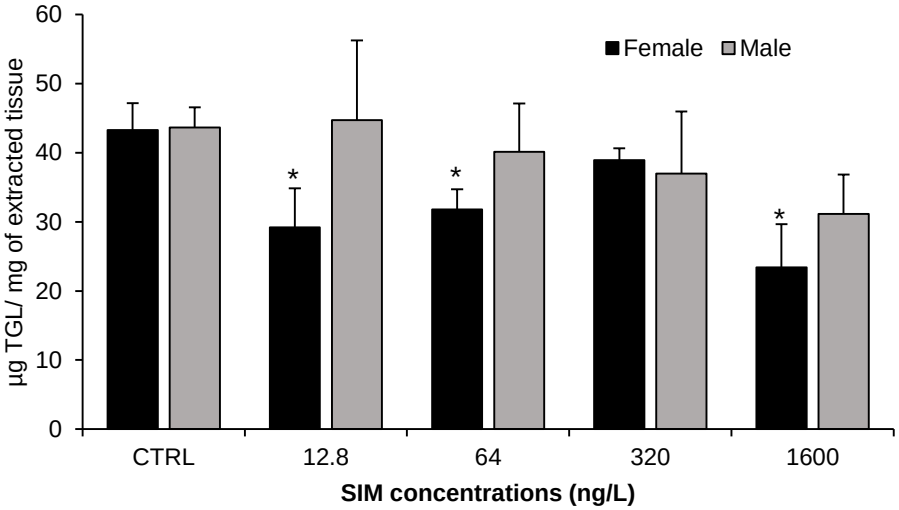

Figure 19. Chronic toxicity effects of SIM on *D. rerio* liver triglycerides content, expressed as µg per mg of extracted tissue, after 90 days of exposure. Error bars indicate standard errors; asterisks (*) indicate significant differences from the control group ($p<0.05$). As no significant differences were found between control and solvent control groups, the data from these two treatments were pooled and referred to as control.

CHAPTER IV.
Discussion

4. Discussion

Hypocholesterolaemic drugs, such as SIM, are designed to produce, in humans, specific biological effects at low concentrations and therefore, once in the aquatic environment, many aquatic taxa might be at risk. Despite the growing number of studies regarding acute toxicity of SIM in aquatic ecosystems, the chronic effects of this contaminant has not yet been properly studied although aquatic organisms are exposed for their entire life to chronic low levels (Santos et al., 2016).

In order to address the current gap of knowledge regarding the effects of chronic exposure of non-target organisms to SIM, the present study aimed at investigating a multilevel biological response in the model organism *D. rerio*, following a chronic exposure (90 days) to environmentally relevant concentrations of SIM. This work integrated key ecologically relevant endpoints such as survival, growth, reproduction and embryonic development and link it with SIM mode of action, focusing in cholesterol and triglycerides content and in the expression of key mevalonate pathway genes. Our results show that chronic SIM exposure induced several disruptive effects at biochemical/molecular levels and in early life stages of *D. rerio*, as well as at some ecologically relevant endpoints. Results also show some sex-dependent differences, suggesting that females and males may respond differently to SIM.

The chronic exposure to SIM in the ng/L range leads to a significant decrease of male weight at the highest exposed concentration (1600 ng/L). Some authors have reported that weight loss is frequently accompanied with decreased cholesterol levels (Cong et al., 2012; Nam et al., 2014). However our study showed that the cholesterol levels of exposed males did not fall as expected. In contrast, the cholesterol levels of exposed female decreased but their body weight remain unaltered. Similar reduction of body weight were reported by Mu et al. (2015) in adult zebrafish exposed to difenoconazole. Thus, the body weight decrease observed here does not seem to be related with cholesterol levels, but may be linked with the effect of SIM on other parameters. However, the origin of body weight decrease in males after SIM exposure, should be better studied. Additionally, significant differences were also observed for females' fecundity. An increase in the number of embryos per female was reported for the highest SIM concentration (1600 ng/L) after 70 days of exposure to SIM. This response may be due to compensatory mechanisms developed during the exposure period as an attempt to counterbalance the effects of SIM. The exposure to SIM could lead the animals to redirect their energy resources to maintain certain body functions in order to cope with the environmental alterations in a phenomenon called allostasis: "the ability to achieve stability through change". Even though this process demands high energy costs, it is able to increase the overall fitness of the animal

(Schreck, 2010), which could possibly be the reason for the improvement of zebrafish fecundity. To further address the effect of SIM in the reproduction, an embryo bioassay was performed with embryos resulting from the parental exposed generation and maintained in clean dechlorinated water. Our results showed an increase of embryo abnormalities for median and lowest SIM concentrations (12.8 and 64 ng/L) with tail abnormalities (curl and/or shortening of the tail) and developmental arrest being the most frequent anomalies. We also observed a significant increase in heart beats for 64 ng/L of SIM. Previous studies demonstrated that SIM exposure was able to produce similar morphologic alterations to those here observed. For instance, Cunha et al. (2016) observed embryos development arrest as well as curled tails, while tail shortening was observed by Campos et al. (2016). There was evidence that zebrafish embryos directly exposed to SIM also present abnormalities in the circulatory system such as pericardial edema (Campos et al., 2016; Cunha et al., 2016). The present study did not record all the detrimental effects reported in the literature. In our experiment, embryos were exposed to SIM only during roughly two hours, meaning that parental exposure might be responsible for a significant amount of embryonic abnormalities. Several detrimental effects were here observed in the embryo bioassay, revealing that harmful effects produced by SIM exposure may possibly be passed to the second generation. Consequently, population level effects might occur, since the observed abnormalities in zebrafish embryos will, most likely affect the fitness of these animals and, consequently their survival. Although the detrimental effects observed at embryonic stages, SIM did not significantly impact Zebrafish key ecological endpoints such as growth and reproduction. It may be hypothesized that a longer exposure time, involving whole life-cycle or multigenerational exposure, at the same environmental relevant SIM concentrations would be necessary to produce effects at other relevant ecologically relevant endpoints. Moreover, taking into account the effects obtained at molecular and biochemical levels, we expect more effects at ecological levels after longer exposures. Multigenerational assays could also be important to better understand the actual impact of the malformations obtained at embryonic development level.

Cholesterol, as the precursor of steroid sex hormones, plays an important role in many essential biological processes, including reproduction (van der Wulp et al., 2013). Additionally, cholesterol has a particularly important role in the membrane as it helps regulate its fluidity and maintains the cell's integrity (van der Wulp et al., 2013). On the other hand, in most animals, triglycerides play an important role in energy storage, being the main source of reliable energy used for growth, reproduction and maintenance of the organisms' biological functions (Vergauwen et al., 2010). Due to its extreme importance, studying the cholesterol and triglycerides content of zebra fish exposed to SIM becomes essential in order to better understand this drug's effect. In fact, our data revealed that

SIM was able to significantly reduce the cholesterol levels in females exposed to 64 and 320 ng/L of SIM. These low levels of cholesterol observed in females exposed to SIM might be the reason for the abnormalities observed in the embryo bioassay. Our results suggest that cholesterol decrease in the parental generation can be associated with morphologic abnormalities on the progeny and potential adverse effects at population level. Triglycerides quantification revealed similar behaviour as cholesterol, female zebrafish levels were significantly decreased in several SIM treatments (12.8; 64 and 1600 ng/L), with the highest concentration, i.e. 1600 ng/L, showing the lower levels. Male zebrafish did not shown any significant differences in triglyceride and cholesterol content but presented slight decrease with the increase of SIM concentrations. It is possible that the decrease of triglycerides and cholesterol reserves in females zebrafish are due to the expenses needed for reproduction, which generally produce high amounts of eggs in a regular basis containing large amounts of lipid reserves (Landgraf et al., 2017). Similar effects on cholesterol and triglycerides levels were already reported by Al-Habsi et al. (2016) after exposing zebrafish to atorvastatin for 30 days. A significant reduction of cholesterol and triglycerides were observed by Al-Habsi et al. (2016) only for females. Interestingly, in the current study, the treatments in which triglycerides decreased (12.8, 64 and 1600 ng/L of SIM) were the same in which the female fecundity increased. It is possible that triglycerides were used as energy source for the previously discussed adaptation mechanisms developed for maintenance of zebrafish reproduction. Sex dependent effects were observed in some of the analysed endpoints on both biochemical and molecular levels. Females seemed to be more prone to biochemical changes, while at molecular level males showed more changes in gene expression.

Cholesterol reduction through the inhibition of the conversion of HMG-CoA to mevalonate by the enzyme HMGCR have been exhaustively studied in humans and other mammalian organisms for more than fifty years since its discovery by Siperstein and Guest (1960). Several studies have demonstrated that HMGCR inhibition by statins led to a decline in MVA synthesis and its downstream products leading to the inhibition of cholesterol biosynthesis. A negative feedback regulation takes place, carried out by SREBP2 activation, which promotes the transcription of HMGCR and LDL receptor, which play an important role in cholesterol synthesis and uptake, respectively (Figure 3). As this pathway is highly conserved through vertebrates, we expect the same mechanisms of action of statins (SIM) occurring in aquatic vertebrates, such as zebrafish (Santos, et al., 2016). Some previous studies with statins in aquatic vertebrates have obtained low levels of cholesterol, as well as the presence of the feedback mechanism regulation at transcriptional levels by presenting increased levels of mRNA expression of genes such as *srebp2, hmgcr* and *ldlr* in a similar way as in mammals.

In order to obtain a more complete analysis of long-term effects of SIM in zebrafish, we also integrated in this study a molecular assessment of several genes expression involved in the mevalonate pathway. Taking into account the literature, we hypothesised that mRNA expression of both *hmgcra* and *srebp2* would increase. In contrast, our results showed that mRNA expression of SREBP2 for males and females maintained its levels near control group or significantly decrease in several SIM concentration for both males and females. As a consequence of the lack of increase of the mRNA expression of *srebp2*, we observed that mRNA content in *hmgcra* significantly decreased for both males and females exposed to 64 and 320 ng/L of SIM. There is no apparent explanation in the literature for this response. Since SREBP2 plays a major role in the feedback regulation of cholesterol which end up increase cholesterol synthesis once their levels have lowered after statin treatment, some authors have discussed possible ways of inhibiting the activity of SREBP2 as a way of lowering cholesterol levels more efficiently. In fact, to the best of our knowledge no studies in the literature have shown the down regulation of *srebp2* and *hmgcra* after statin treatment, most of the studies available report up regulation of these genes when cholesterol levels decreased due to the natural feedback mechanism of the MVA pathway. Most studies regarding SIM MOA in vertebrates which obtained these results are based on short acute exposures. However it is possible that chronic exposures might result in different effects (Al-Habsi et al., 2016; Egom & Hafeez, 2016; Xiao & Song, 2013). However it is also possible that the observed effect could be a result of fluctuations in gene expression or feedback mechanism at the time-point the sampling was performed. More research should be performed in order to better assess long-term effects of SIM in *srebp2* and clarify as well if there is another pathway that might be involved on its regulation.

As a consequence of *hmgcra* down regulation, it is likely that genes downstream in the pathway most likely should be down regulated as well, which was observed for *cyp51.* For being the key factor in the hydrogenation of 7-dehydrocholesterol, the precursor of cholesterol (Figure 3), *dhcr7* expression was also assessed. However, even though cholesterol levels suffered a significant decrease, *dhcr7* mRNA transcript levels remained unaltered. There is a clear lack of knowledge necessary to explain the gene expression profile observed after long-term exposure to SIM. Therefore, expression of additional genes related to cholesterol biosynthetic pathway should be assessed. Genes related to transport, efflux and degradation of both SIM and cholesterol should also be quantified since these are some of the mechanisms that may also influence cholesterol levels, as well as SIM accumulation in the organism.

Our results on the mRNA transcript levels of *srebp2* and *hmgcra* seem to be different to data recorded in the literature. However there are also some studies revealing

contrasting results around statins ability to decrease cholesterol synthesis as well. For instance, Goldberg et al. (1990) found out that patients used lovastatin to treat high cholesterol levels were not able to reduce cholesterol synthesis. Kallen et al. (1999) even revealed to have observed increased cholesterol synthesis when treating his patients with pravastatin. Raghow (2017) stated that although a high amount of research has been done over the last decades in cultured cells and experimental animals, the mechanistic details underling statins effects *in vitro* are still poorly understood, revealing urgent necessity of *in vivo* testing of these pharmaceuticals.

Some of our most relevant results showed a non-monotonic dose response curve (NMDRC) with a U-shaped curve (for cholesterol quantification and gene expression) and inverted U-shaped (for embryonic abnormalities). A NMDRC is mathematically defined as a response where the slope of the curve changes sign from positive to negative, or vice-versa in the range of doses examined (Kohn & Melnick, 2002). The mechanisms that cause this non-monotonicity are currently unknown. For many years, it was assumed that contaminants exhibited linear monotonic responses, assuming that "the dose makes the poison" (Vandenberg et al., 2012). However in the last years the occurrence of NMDRC has been more frequently documented (Andrade et al., 2006; Barros et al., 2017; Crépeaux et al., 2016; Do et al., 2012; Faigón et al., 2014; Kim et al., 2014). Calabrese and Baldwin (2016) stated that NMDRCs might occur more frequently than it is expected. The reason behind this is that NMDRCs based in adaptation mechanisms are generally dependent of exposure time and range of concentrations tested. It was already known that these kind of response curves generally occur at low-level concentration. However, time of exposure can also greatly influence their occurrence, because adaptation mechanisms need time to develop and it is difficult to predict the time during which the response will occur and/or be maintained, meaning that in the majority of studies performed exposure time may be too brief or too long in order to detect this type of NMDRC. There are no records of this type of response for SIM or other statins until the present study. The reason behind it might be that in most cases, studies with statins had shorter exposure periods, as well as higher concentrations when compared to the conditions of our experiment. It is also possible that the non-monotonic curves obtained in this study may be a result of the development of an adaptive response to overcompensate the effects of SIM at the highest SIM concentrations tested. Muskak (2016) argued that adaptation mechanisms may produce NMDRCs after exposure to certain contaminants and that this kind of response may be transitory or continue to operate during long periods of time upon low-level chronic exposures.

Our experiment revealed several interesting and new results not yet reported in the literature for this class of pharmaceuticals. It is highly necessary to perform more stud-

ies in both aquatic vertebrates and invertebrates in order to clarify the mode of action behind SIM long term exposures, which might be more relevant than expected.

CHAPTER IV.
Conclusion

Conclusion

Our study revealed that SIM was able to affect the MVA pathway in the aquatic vertebrate *D. rerio*. Despite SIM significantly affected the majority of our tested endpoints, i.e. ecological, biochemical and molecular endpoints, many questions remain about the mode of action of statins after long-term exposures. Our study reported for the first time, several non-monotonic dose-response curves for statin exposure at biochemical and molecular endpoints. Results on the embryonic assay raised great concern regarding the future of aquatic vertebrate' populations. The environmental realistic concentrations assessed in our study revealed several abnormalities in the embryos of animals which had their cholesterol levels significantly decreased. It is also important to note that in this experiment was based in a partial life-cycle exposure with a duration of 90 days. Therefore, a multigenerational exposure to SIM should be performed in order to simulate a more realistic scenario of environmental exposure to SIM and better understand the potential effects at individual and population levels, as well as provide additional insights in the understanding of the responses obtained in the present study and their implications.

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